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REVIEW

Location – positioning Tregs to the right place at the right time

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After a fruitless pursuit for suppressor cells spanning 1970s to mid-80s, the identification of $CD4^+CD25^+$ T cells as a specific T-cell lineage with immune regulatory function in the 1990s has revived the theory of active immune tolerance and uncovered a brand-new avenue for immunologic research. Tregs have been shown to play a crucial role both in health and diseases. Whilst tremendous advance has been made in our understanding of the expanding varieties of effector mechanisms exploited by Tregs, the migration phenotypes as well as the anatomic sites where Tregs exert immune regulation are scarcely investigated. Migration of Tregs to either the lymph nodes or peripheral tissues has been shown to be exclusively indispensable in Treg-mediated immune regulation in a variety of experimental models with specific gene-targeted mice. The once seemingly paradoxical findings are partly reconciled by later discovery of various Treg subsets with distinct migration/homing property as well as the inflammatory stage when Tregs come into play along an immune reaction. In our recent study, we investigated the migration characteristics of Treg cells by using the endothelial cell-based shear-stress flow assay that resembles the intravascular blood flow system. We found that both FoxP3-expressing Tregs and anergic T cells generated by blockage of costimulation factors, CD80 and CD86, exhibited a significantly decreased adhesion to endothelial cells as compared to antigen-activated effector T cells (66~88 % reduction). The less migration phenotype hinted inefficient tissue trafficking of the Tregs and suggested the lymph nodes as the anatomic site where Tregs optimally exerted immune regulation. To this speculation, an essential role of Treg lymph node positioning in exerting immune suppression was demonstrated by the inability of adoptively transferred Tregs to prevent footpad inflammation after blockage of lymph node entry of these Tregs by CCR7 or CD62L Ab. Therapeutic modality targeting leukocyte migration has been a mainstay alternative for immunologic diseases. Important messages have arisen for treatments of this kind and Treg-based cell therapy that whilst inflammatory response can be harnessed by modulating the migration property of leukocytes, the relationship between Treg migration phenotypes and their immune regulatory function should always be taken into consideration.

Keywords: Regulatory T cells; immune regulation; leukocyte migration

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Induction of peripheral tolerance as a therapeutic strategy for various immunological diseases has gained intense attention in the past two decades [1, 2]. Among the mechanisms identified in the operation of peripheral tolerance, Tregs are the most well-known key player [3, 4]. Tregs have been shown to exert immune suppression in a cell-contact dependent manner (Treg/APC or Treg/T-cell interactions), through the expression of PD1, CTLA-4, and TGF-β, or via secretion of various humoral mediators, such as granzyme B, IL-10, IL-35 and etc [5-7]. Additional molecular mechanisms include the CD39/CD73 ectonucleotidase that regulates ATP/AMP metabolism and adenosine production [8-10] as well as immune-regulatory microRNAs, such as miR-146a [11] (Fig 1A). Despite cumulating data have unraveled the mechanisms underlying Treg-mediated immune regulation, relatively scarce attention has been paid to where the immune suppression takes place in vivo. The issue arisen from this unsolved question is the feasibility and effectiveness of exploiting Treg cells in cell therapy for immunologic diseases.

Leukocyte migration during inflammation

Migration of leukocytes from the blood vessels into peripheral tissues is a crucial process both during immunosurveillance and inflammation. This response is orchestrated by sequential steps of leukocyte rolling, activation, firm adhesion to the endothelial cells (EC) and migration through the inter-endothelial junction and the vascular basement membrane [12, 13]. These cellular events require adhesive interactions between leukocytes and the EC and are largely mediated by selectin and integrin family of the adhesion molecules. As much as the magnitude of an inflammatory response could be modulated by harnessing the migration activity of the leukocytes, it is also theoretically feasible that immune tolerance can be achieved by targeting leukocyte migration.

LN vs tissue localization of Tregs matters in immune regulation

The long-held dogma of an immune response is characterized by recognition of foreign antigens in the periphery by antigen-presenting cells (APCs), such as the dendritic cells (DCs), followed by trafficking of these cells back to lymph nodes (LNs) to present processed antigens to T cells. The T cells, being activated by presented antigens, proliferate, and after providing help for B-cell antibody production, migrate out of LNs to enter the peripheral tissues where they secret cytokines to propagate the inflammatory cascade and execute cytotoxic effects. Treg cells are present not only in lymphoid organs but also in non-lymphoid peripheral tissues both at steady state and during inflammation. It is therefore equally logic for Tregs to exert their immune suppressive effects in either the draining LNs or the tissues, i.e. during immune induction or at the effector phase of an immune response. However, contradictory and mutually exclusive results have been shown by the studies using different experimental settings [14-17].

CD62L and CCR7 are required for leukocyte LN trafficking. Blockage or deficiency of either CCR7 or CD62L prohibits LN positioning of leukocytes, including Tregs. In an allograft tolerance animal model, Tregs expanded selectively in the LNs of tolerant animals, but not in other anatomic sites or in animals with graft rejection. CD62L blockage of Treg LN localization led to graft rejection, whereas Treg LN sequestration by sphingosine 1-phosphate receptor agonist enhanced graft survival [18]. In line with these findings, previous studies have also showed that the $CD62L^+$ LN-homing Tregs exerted suppressive function far better than their $CD62L^-$ tissue-homing counterparts, despite the two subpopulations were equally suppressive in vitro [19;20].

The same indispensable role of LN positioning in Treg-mediated immune suppression was demonstrated in CCR7-deficient mice [21]. Whist enhanced contact hypersensitivity was induced in these animals, the pathology was rescued by adoptive transfer of WT Tregs that were capable of LN trafficking [22]. Additional evidence supporting a crucial role of Treg LN positioning in immune tolerance comes from studies of autoimmune disease where preferential enrichment of Ag-specific Tregs in regional LNs was found in normal healthy mice [23]. These studies went on to demonstrate that strategic localization of Ag-specific Tregs in the draining LNs enabled persistent inhibition of pathogenic T-cell expansion selectively in regional LNs but not elsewhere [23, 24].

In our recent study, we found a less migratory phenotype of the Treg cells. $CD4^+CD25^+$ Treg cells and anergic T cells generated in vitro by blockage of costimulation factors exhibited significantly decreased adhesion to either activated endothelial monolayer or ICAM-1/ E-selectin-coated surface compared to activated effector T cells (Fig 2). The less migratory capacity of the regulatory T-cell population prompted our speculation of the in vivo positioning of Tregs for optimal immune regulation. It is conceivable that localization of Tregs in the lymphoid organs, instead of the peripheral tissues, would provide an optimal condition for cell contact-dependent immune regulation, and that at the priming or induction phase of an immune response. In addition, the low migratory capacity of the Tregs during inflammation would be insufficient to support adequate trafficking of these cells into inflammatory sites to exert

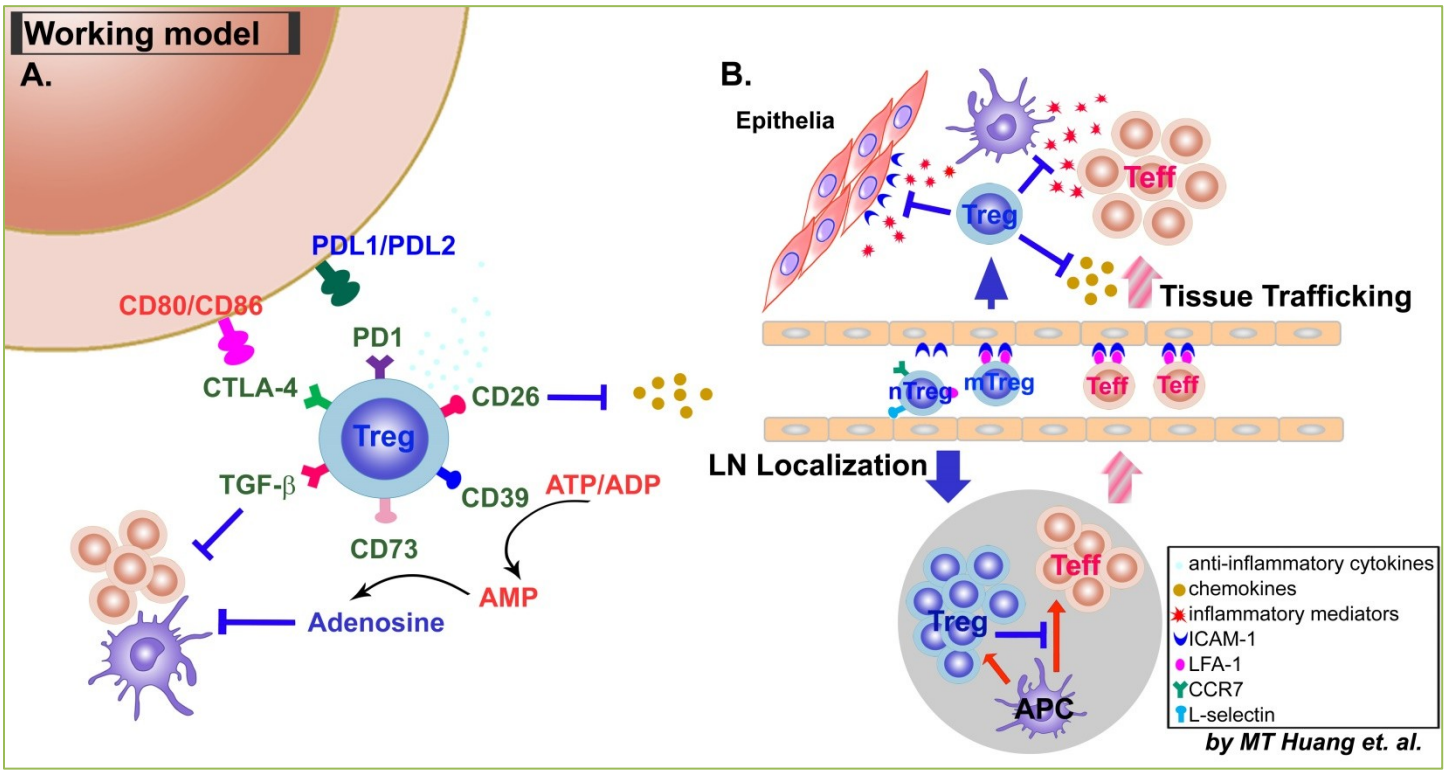

Figure 1. Working model depicting Treg migration pattern and their immunosuppressive effects. (A) Mechanisms of Treg-mediated immune suppression: direct cell-cell contacts deliver inhibitory signals *via* PD1, CTLA4, TGF-β, etc; secretion of immunoregulatory cytokines; metabolism of ATP/adenosine; regulation of chemokine and adhesion molecule expression and hence tissue trafficking of Teff; (B) Treg migration phenotypes and immune regulation. Tregs exert immune suppression at either the LNs or peripheral tissues. Individual Treg subsets expressing different profile of chemokine receptors and adhesion molecules exhibit distinct migration property. Whilst the naïve-like Tregs (nTreg) "home" to LNs, the memory-like Tregs (mTreg) preferentially migrate to inflamed tissues. Reprinted with permission [25].

immune regulation. In concordant with this speculation, we found that CCR7 or CD62L blockage of Treg LN entry abrogated the immune suppressive effects of the Tregs, suggesting an essential role of Treg LN positioning in immune suppression which should be taken into account in Treg-based cell therapy [25].

On the other hand, there are studies showing a crucial role for tissue localization of Tregs in immune regulation. In fucosyltransferase VII-deficient mice, migration of leukocytes to peripheral tissues was impaired due to lack of P-/E-selectin ligands. Tregs from fucosyltransferase VII-deficient mice failed to migrate to inflamed tissues and were unable to suppress skin inflammation as the WT Tregs did [16]. However, opposite outcome of impaired Treg tissue localization was demonstrated by studies using β7 integrin-deficient mice. Although β7 integrin-deficient Tregs were incapable of immigrating to the intestines, they were nonetheless fully competent in preventing intestinal inflammation [17]. Adding complexity to these results, studies using various CCR-deficient mice proposed a sequential migration pattern of Treg cells in exerting immune suppression. These studies showed that Tregs were first recruited to the tissues and activated during inflammation before trafficking back to draining LNs to suppress immune activation [26].

These seemingly contradictory results clearly demonstrate that both lymphoid organs and peripheral tissues can be the anatomic sites where Tregs suppress inflammatory responses [14-17]. These data also reflect the in vivo scenario that Tregs exert immune regulation at both the induction and effector phases of the immune reaction and lead to the revelation of a "division of labor" for Tregs - different Treg subsets with distinct migration phenotypes [14; 16, 20]. There are at least two Treg subpopulations - the re-circulating naïve-like and the inflammation-seeking effector-like Tregs - each defined by distinct homing/transmigration characteristics and differential expression of chemokine receptors and adhesion molecules (Fig 1B). It is therefore crucial for proper positioning of individual Treg subsets of distinct homing/transmigration property that might be selectively expanded along the course of an inflammatory reaction under various experimental settings.

Tregs-EC crosstalk over leukocyte migration

Induction of tolerance has been associated with thwarted

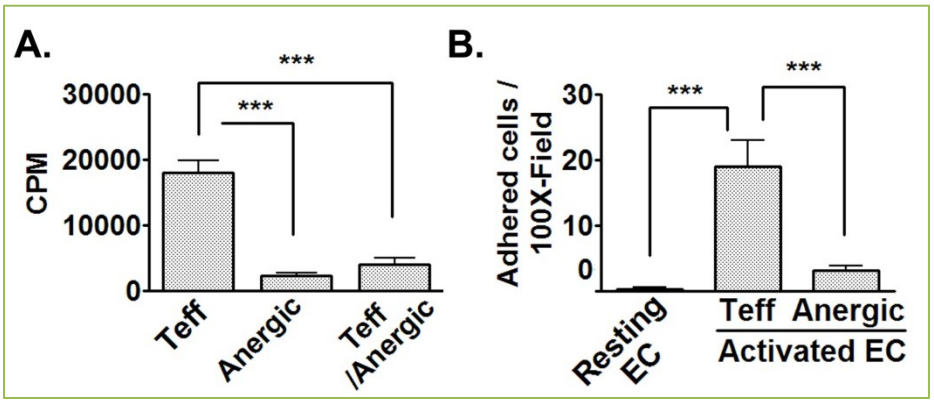

Figure 2. Anergic T cells exhibit a less migratory phenotype than their effector counterparts. $CD4^+$ T cells were isolated from $OVA_{323-339}$-TCR transgenic DO11.10 mice by magnetic beads-conjugated antibodies as per manufacturer's negative selection protocol (Miltenyi Biotech). Activated effector T cells (Teff) were generated by incubating isolated $CD4^+$ T cells with irradiated APCs at the presence of OVA (200 g/mL) for 3 days. Anergic T cells were induced by the above culture condition with the addition of anti-CD80 and anti-CD86 blocking Abs (10 g/mL). At 72hr after culture, cells were harvested for further assays. (A) CD80/CD86 blockage induced immunosuppressive anergic T cells. Immunosuppressive effect of the anergic T cells were examined by co-incubating the anergized T cells with freshly isolated DO11.10 $CD4^+$ T cells and irradiated APCs at the presence of OVA. Cell proliferation was detected by H^3 incorporation assay at 72 hr of culture; (B) Anergic T cells are less migratory than activated Teff under flow assay. Cells harvested after 72-hr culture were enumerated and flowing through cytokine-stimulated or resting endothelial monolayer under physiologic shear stress of 1.5 dynes/cm^2 and cell adhesion was enumerated as described [25].

T-cell migratory ability and retention of T cells at the site of tolerance induction [27-29]. Intracellular signaling downstream of chemokine receptors is controlled by the regulators of G-protein signaling (RGS) family and RGS-1 is known to inversely correlated with cell migration capacity. To this end, Tregs were shown to exhibit a less migratory phenotype than naïve T cells and expressed higher levels of RGS-1 than naïve T cells [28, 29]. Furthermore, in the context of leukocyte migration during inflammation, impeded trafficking of inflammatory leukocytes into peripheral tissues inevitably abrogates the inflammatory cascade. One mechanism reportedly exploited by Tregs to dampen inflammatory response is via regulating the migration of inflammatory leukocytes into inflamed tissues. Mechanistically, these effects are due to degradation of chemokines by CD26, the dipeptidyl peptidase IV, specifically expressed by Tregs and anergic T cells, which led to decreased adhesion-dependent leukocyte trafficking driven by chemokines [27, 30].

Leukocytes undergo phenotypic and functional changes after migration through the EC monolayer [31], it is therefore equally possible that leukocytes crosstalk with the EC or epithelia to modulate their phenotypes and functions. Although there are only few researches addressing the interaction between Tregs and EC or epithelia during inflammation, bidirectional crosstalks between EC and the Tregs have been shown. In one of these studies, CD8+ FoxP3+ suppressor T cells were shown to induce tolerogenic EC through mechanisms involving induction of the inhibitory receptors, immunoglobulin-like transcripts (ILT)-3 and ILT-4, and down-regulation of costimulatory and adhesion molecules on the EC. The tolerogenic EC in turns suppressed activation of T helper and cytotoxic cells and elicited differentiation of new CD8+ FoxP3+ suppressor T cells [32, 33]. Another recent study addressed the same issue by using β7 integrin-deficient mice. In contrast to previous studies showing suppression of T cell-mediated colitis in Rag2-/- mice by β7-deficient Tregs [17], this study found aggravated DSS-induced colitis in β7 integrin-deficient mice that can only be rescued by WT Tregs but not β7-deficient Tregs [34]. In this study, WT Tregs were shown to migrate to the intestine and down-regulate epithelial expression of ICAM-1, which hampered recruitment of inflammatory cells and prevented progression of colitis. However, β7 integrin-deficient Tregs failed to migrate to the intestine to exert this effect. The contradictory results between the two colitis models can be explained by nature of the inflammatory response elicited in the models, e.g. Ag-driven adaptive vs innate inflammation and hence the distinct roles or mechanisms of regulation exploited by Tregs under different inflammatory conditions.

In clinical setting, therapies targeting leukocyte migration by using blocking antibodies against integrins or integrin ligands have been approved or in various phases of clinical trials for inflammatory bowel disease (IBD) [35, 36]. It is

noteworthy that aggravated colitis was ever observed in a small percentage of ulcerative colitis patients treated with α4β7 Ab (vedolizumab) [37] or β7 integrin Ab (etrolizumab) [38]. Considering the role of Treg migration phenotypes in their immune suppressive function as well as the regulatory effects of Tregs over the EC and epithelial cells, it is possible that excessive inhibition of integrin function prevents tissue trafficking of not only the inflammatory leukocytes but also immune regulatory Tregs that outweighs the beneficial effects of decreased inflammatory cell recruitment into the intestine mucosa and eventually caused the exacerbation of colitis.

Conclusions

Cell-based therapy and therapeutic measures harnessing leukocyte migration are two main themes of optimistic potential to bring basic immunology bench works into bedside treatments. Tregs are in the center stage of this new therapeutic modality due to their immune regulatory function and the feasibility to be modulated to work for both sides, *i.e.* induction of tolerance or breakthrough. We and others have demonstrated a crucial role for Tregs to be positioned in the right anatomic sites for optimal immune regulation. Individual Treg subsets with distinct migration phenotypes, the nature and the stage of the immune response, and the intervention strategies to be undertaken are keys to success. As much as therapies aimed at targeting leukocyte migration have gained some success in IBD and application of this new therapeutic modality in other inflammatory diseases to come, the impact attributed to alteration in Treg migratory phenotype and migration-related immune regulation as exerted by Tregs during immune suppression should not be overlooked.

Conflicting interests

The authors have declared that no conflict of interests exists.

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Abbreviations

Tregs: regulatory T cells; EC: endothelial cells; APCs: antigen-presenting cells; DCs: dendritic cells; LNs: lymph nodes.

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